Deciphering Spatial Heterogeneity in Bacteria Social Behaviors

1st Project Proposal

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Background

Bacteria, like human, exhibit social behaviors despite their microscopic nature. Such social behaviors are primarily mediated by communication mechanisms, notably quorum sensing (QS), which enables bacteria to coordinate their activities based on population density (Dunny et al., 2008; Miller & Bassler, 2001; Xavier & Foster, 2007). QS involves the production, release, and detection of extracellular signaling molecules known as autoinducers. When the concentration of autoinducers reaches a certain threshold, bacteria trigger collectively behaviors that are beneficial for their community, including biofilm formation, the production of virulence factor, and the development of antibiotic resistance (Li & Tian, 2012; Nadell et al., 2009; Rutherford & Bassler, 2012; Xavier & Foster, 2007). This sophisticated communication mechanism is vital for bacterial survival and proliferation, particularly in fluctuating environments. It allows bacterial population to function as cohesive units rather than as isolated individuals, hence also playing a crucial role in community assembly and stability (Even-Tov et al., 2016; Mukherjee & Bassler, 2019).

Importantly, bacteria within communities are not homogeneously distributed. Instead, they display spatial arrangements influenced by nutrient availability, physical barriers, and environmental gradients. These distinct spatial patterns can profoundly affect bacterial social interactions and alter their behaviors, leading to formation of subpopulations with different functions (Dal Co et al., 2019). For instance, the diffusion limitations of autoinducers and specific cellular arrangements within a community can result in subpopulations of cells that are either QS-active or inactive (Mukherjee et al., 2019). Furthermore, spatial heterogeneity is intricately linked to temporal complexity. Bacteria regulate their social behaviors dynamically over time, adapting to environmental conditions during the development of mature communities. This fine-tuned regulatory system not only enhances their ability to respond to fluctuating environments but also serves as a bet-hedging strategy to increase survival chances under uncertain conditions (Jayakumar et al., 2022). Understanding the temporal and spatial dynamics of bacterial social behaviors during community formation – such as the development of microcolonies and biofilms – is crucial for advancing our knowledge of bacterial ecology. Moreover, this knowledge could pave the way for innovative strategies to manipulate microbial behaviors, potentially offering new solutions for addressing challenges like biofilm-associated infections or antimicrobial resistance.

Aim of the project

The goal of my PhD is to unravel the temporal and spatial intricacies of bacterial social behaviors, with a primary focus on quorum sensing (QS), during the development of mature bacterial communities. These communities range from microcolonies to biofilm, representing different stages in the formation of mature bacterial communities. Each stage is characterized by complex and distinct architectures, as well as varying interaction patterns mediated by QS. By studying these developmental stages, we can not only gain insights into the spatial organization of mature communities like biofilms but also better understand the spatial arrangements in early phases, which play a crucial role in shaping the composition and dynamics of the community later. By investigating the interplay between QS-mediated communication, spatial organization, and social interactions, I aim to enhance our understanding of the ecological and evolutionary significance of bacterial communication and cooperation, thereby providing deeper insights into the assembly and stability of bacterial communities. These insights could have broad implications, enhancing our knowledge of

complex microbial ecosystems while informing the design of novel strategies to combat antibiotic resistance and control pathogenic bacteria.

Planned Experiment

To address my research goals, I have chosen ***Pseudomonas aeruginosa*** as my model organism. *P. aeruginosa* is a Gram-negative, opportunistic human pathogen with a highly intricate QS regulatory network as shown in Figure 1. It employs three QS systems: *las*, *rhl*, and *pqs*. The *las* and *rhl* systems both use acyl-homoserine lactones as the backbone for their autoinducers and operate through a cascade involving three key genes: the signal synthase gene, receptor gene, and product genes. The *pqs* system, in contrast, utilizes quinolones as autoinducers and features a more complex gene cascade. Together, these systems regulate the expression of hundreds of genes in response to increasing cell density, orchestrating processes ranging from virulence factor production to biofilm formation.

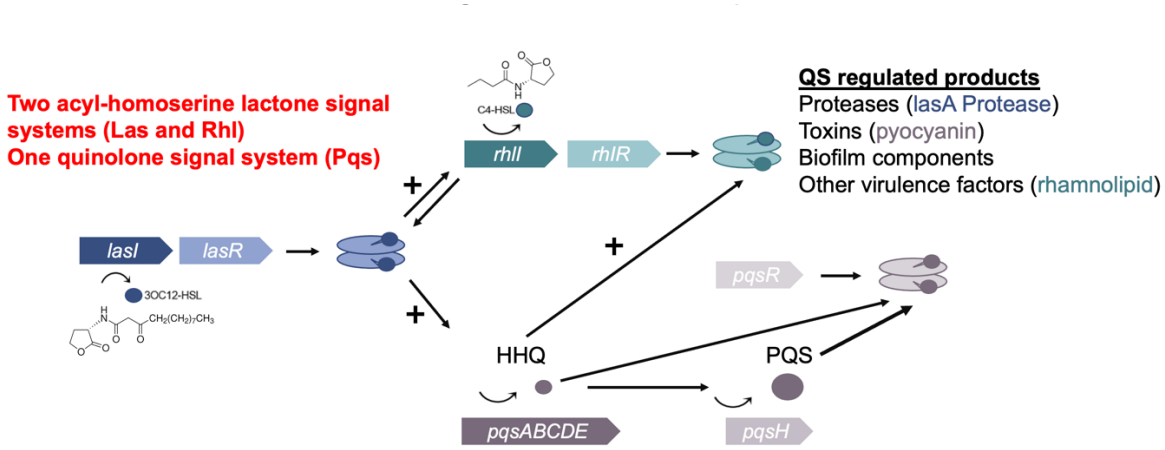


Figure 1 Schematic Diagram of QS in Pseudomonas Aeruginosa (modified from Miranda et al., Adv Exp Med Biol, 2022)

I aim to explore the spatial heterogeneity of QS gene expression in *P. aeruginosa* and its influence on the downstream expression of QS-regulated product genes. Furthermore, I am interested in exploring how QS regulates collective bacterial behaviors in polymicrobial conditions, particularly within mature communities such as biofilms. To address these objectives, I have outlined three interconnected projects below, each designed to provide complementary insights into the mechanisms of QS and its ecological significance.

Project 1: to investigate how spatial organization within microcolonies influences QS signal propagation and response at the single-cell level.

- Dissecting correlations between spatial organization and single gene expression in *las*, *rhl* systems (**Finished**)
- Exploring gene co-expression and distinct QS modalities of cells within communities (**Ongoing**)

Project 2: to utilize innovative microchip systems (2. Microchip Systems) to explore and manipulate social behavior in structured environments.

- Exploring how the QS system of *Pseudomonas aeruginosa* facilitates clustering or grouping when the bacterial cells have mobility

    - Introduce oxidative stress or exogeneous AI to observe their behaviors changes
- Investigating spatial aggregation of collective behaviors in bacterial competitions
    - Explore how spatial organization influences QS and the regulation of virulent factors in both **contact-dependent (e.g., T6SS)** and **non-contact (e.g., Phenazine)** bacterial interactions

Project 3: to explore collective behaviors in more mature bacterial communities using Confocal Laser Scanning Microscopy

- Disentangle the 3D spatial patterns of collective behaviors in complex bacterial community
- Investigate the roles of collective behaviors in community assembly and stability

A short compilation of the envisaged methods

1. Microcolony Growth on Agarose Patches

Microcolony growth on agarose patches will be used to investigate structured bacterial communities. This setup allows precise observation of the spatial arrangement and microenvironment of bacterial cells. Using fluorescent-tagged reporter strains, the spatial distribution and temporal dynamics of QS activation will be monitored. High-resolution and time-lapse microscopy, combined with quantitative image analysis, will enable the visualization and quantification of QS activity at the single-cell level. This approach will provide insights into how cell positioning and local cell density affect QS signal distribution and response. Additionally, manipulation using QS signal analogs will be performed to assess their impact on QS dynamics within structured communities.

2. Microchip Systems

Pore network microchip systems offer a robust platform to study QS and other social behaviors in highly controlled microenvironments while maintaining bacterial cell mobility. In this project, I will employ pre-designed microchip systems to study and manipulate bacterial interactions. We have two custom-designed microchip systems as shown in Figure 2. Both systems feature arenas for introducing bacterial strains, with a maze-like structure consisting of numerous chambers connected by micro- and nano-scale channels, enabling the distribution of bacteria and small molecules. Additionally, in design 1, two reservoirs are incorporated, allowing the introduction of specific molecules into the system.

These custom-designed microchip systems create structured bacterial habitats with defined geometries and molecular gradients. Coupled with high-resolution microscopy, the system enables real-time monitoring of QS activity under diverse environmental conditions. By integrating microchips with advanced imaging techniques, the spatial and temporal aspects of bacterial social interactions can be dissected with high precision.

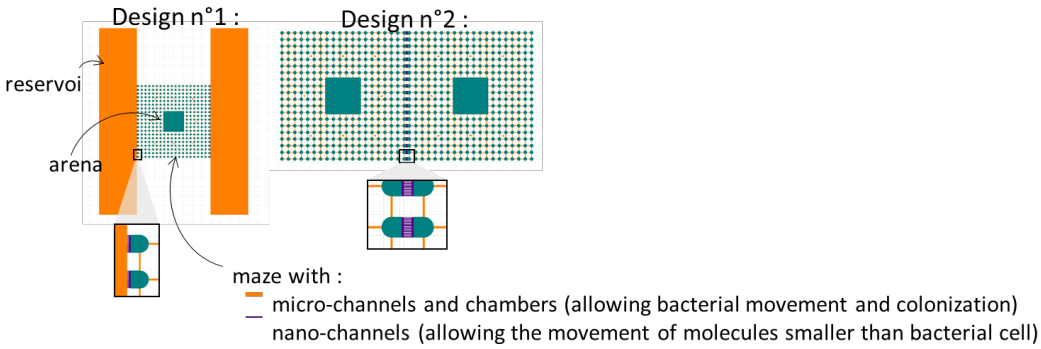


Figure 2 Scheme of Microchip Systems

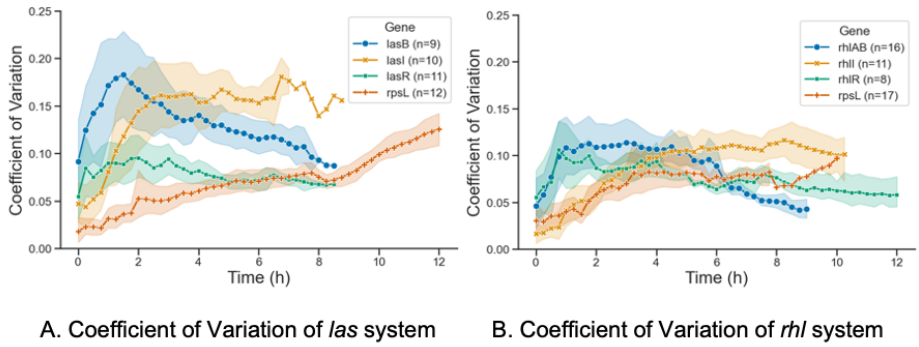
3. Confocal Laser Scanning Microscopy to study biofilm

Confocal laser scanning microscopy (CLSM) provides a high-resolution imaging technique to explore biofilm architecture and QS dynamics in three-dimensional space. By employing fluorescent dyes and genetically encoded reporters, this method enables visualization of biofilm structural organization and QS signal distribution. Time-lapse imaging will allow tracking of biofilm development and QS activation over time, shedding light on the interplay between biofilm heterogeneity and QS signaling. Furthermore, CLSM can be combined with image processing algorithms to quantitatively analyze key biofilm properties, such as cell density, thickness, and matrix composition. This approach will be instrumental in understanding how biofilm structure influences QS-mediated behaviors and responses to environmental perturbations.

Preliminary results

1. Heterogeneity in QS Gene Expression

Our results reveal that receptor gene expression (*lasR*) at the single-cell level remains consistently strong and homogeneous within microcolonies during the onset of quorum sensing, whereas signal gene expression (*lasI*) exhibits notable variability (Figure 3A). This heterogeneity in signal production likely contributes to the observed variation in the expression of downstream product genes. In the *rhl* system, this phenomenon is less pronounced, likely due to the generally weaker fluorescent signal intensity (Figure 3B).



A. Coefficient of Variation of *las* system B. Coefficient of Variation of *rhl* system

Figure 3 Coefficient of Variation over time

To investigate further, we analyzed the spatial autocorrelation of each gene. Signal gene expression exhibited a more random spatial distribution, while receptor expression showed a clustered arrangement. Interestingly, we identified "**signal hotspots**" during the onset of quorum sensing (Figure 4), with hotspot areas for *lasI* being larger compared to *rhlI*. Within these hotspots, a mean of 42.75% (*lasI*) and 12.93% (*rhlI*) of the hotspot areas could be attributed to lineage effects (**3. Lineage Effects in QS Gene Expression**), underscoring the significant role of cellular inheritance in shaping spatial expression patterns.

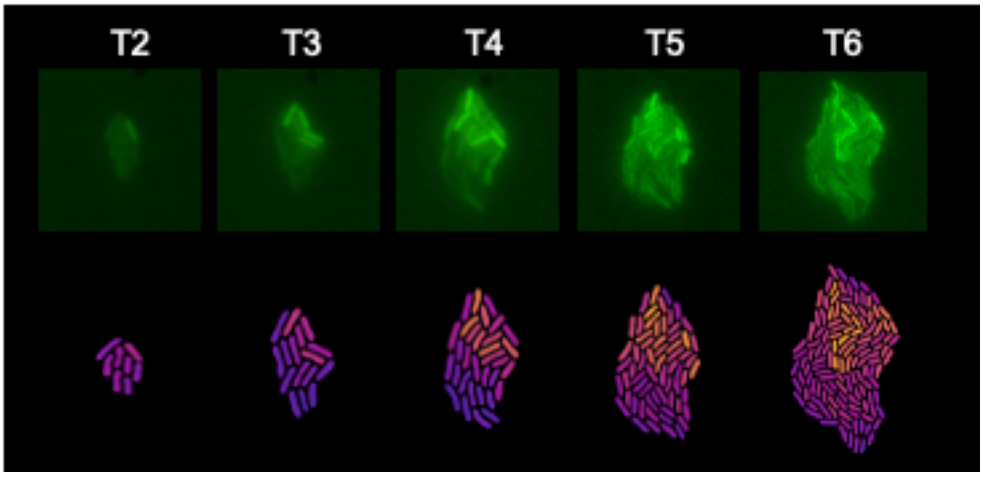
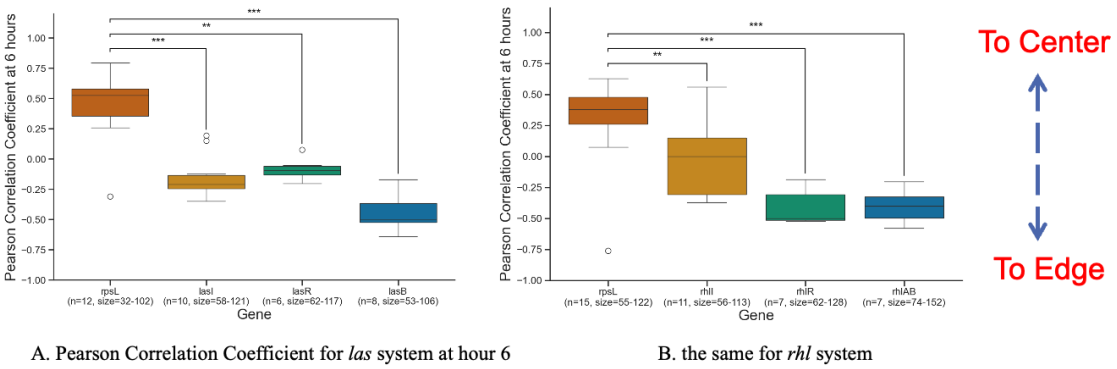


Figure 4 The first line shows an examples of lasI hotspot formation between hour 2 and hour 6; the second line are the heatmaps generated from the original GFP channel images

2. Spatial Patterns of QS Genes vs. Housekeeping Genes

We compared the spatial expression patterns of QS genes with those of the housekeeping gene *rpsL*. By analyzing the Pearson correlation between gene expression and the distance of individual cells to the microcolony edge, we observed that QS genes exhibited distinct spatial patterns compared to *rpsL* (Figure 5). Specifically, QS gene expression showed more negative correlations with distance from the edge, suggesting that QS expressions are more upregulated towards the edge of the microcolony. In contrast, *rpsL* displayed a positive correlation, suggesting a different spatial regulation pattern.

Our assumption is that for *rpsL*, cells located near the microcolony center were surrounded by more neighbors, potentially resulting in higher metabolic activity. In contrast, QS genes displayed the opposite trend, likely due to the diffusible nature of QS signals. Furthermore, we observed that the product genes *lasB* and *rhlAB* demonstrated the most distinct spatial patterns compared to both the housekeeping gene and other QS components, such as signal and receptor genes.



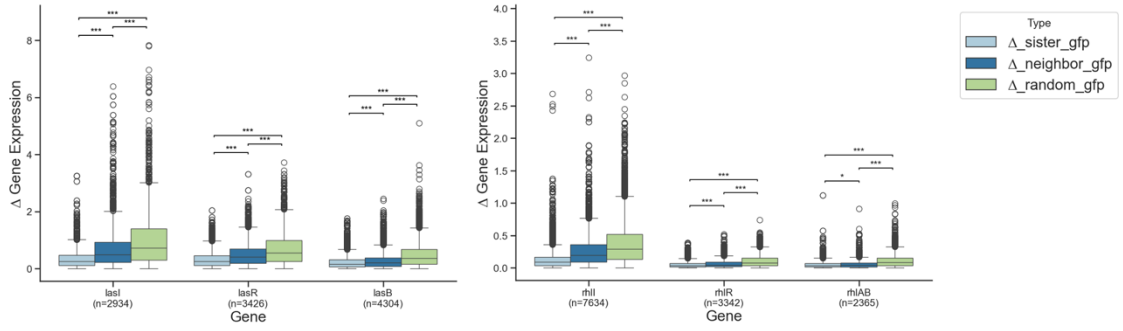
A. Pearson Correlation Coefficient for *las* system at hour 6

B. the same for *rhl* system

Figure 5 Pearson Correlation Coefficient for QS genes expression and distance to colony edge at hour 6

3. Lineage Effects in QS Gene Expression

We disentangled the contributions of lineage and local environment to QS gene expression by comparing the similarities between sister cells, neighboring cells, and random cells within microcolonies. A remarkably strong lineage effect was observed across all QS genes, with sister cells showing the highest similarity in expression levels. Neighboring cells also exhibited some degree of expression similarity, whereas random cells displayed the greatest dissimilarity (Figure 6). These findings emphasize the influence of epigenetic inheritance on QS gene expression within microcolonies.



A. *Las* genes expression similarities between sister, neighbor and random cells

B. the same for *rhl* genes

Figure 6 Variation between sister, neighbor and random cells within microcolonies

4. Predicting QS Activation Using Machine Learning

Using our extensive dataset of single-cell information—including gene expression levels, cell morphology, and local environmental factors—we employed a Random Forest Regressor to predict the peak signal (*lasI*) intensity during a cell's lifespan, a critical marker of QS activation. We incorporated over 16 features, such as microcolony batch information, the presence of exogenous autoinducers, cell morphology (e.g., cell length and aspect ratio), and local environmental factors like neighbor and sister cell expression levels.

Our model achieved an accuracy of 71%, with the intensity peak of sister cells emerging as the most significant predictor, explaining 89.1% of the variation in signal intensity. These results further support our earlier findings, demonstrating that QS gene expression is predominantly influenced by epigenetic inheritance, with local environmental factors, including neighboring cells, playing a secondary role.

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